

Ovigerous-Hair Stripping Substance (OHSS) in an Estuarine Crab: Purification, Preliminary Characterization, and Appearance of the Activity in the Developing Embryos

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Abstract. Ovigerous-hair stripping substance (OHSS) is an active factor in crab hatch water (*i.e.*, filtered medium into which zoea larvae have been released). This factor participates in stripping off the egg attachment structures (*i.e.*, egg case, funiculus, and the coat investing ovigerous hairs) that remain attached to the female's ovigerous hairs after larval release. Thus this activity prepares the hairs for the next clutch of embryos. OHSS activity of an estuarine crab, *Sesarma haematocheir*, eluted as a single peak on molecular-sieve chromatography, but this peak still showed two protein bands at 32 kDa and 30 kDa on SDS-PAGE. The two protein bands stained with a polyclonal antiserum raised to the active fractions from molecular-sieve chromatography. Moreover, antibodies purified from this polyclonal OHSS antiserum also recognized both the 32-kDa and 30-kDa bands. OHSS immunoreactivity and biological activity were associated with the attachment structures that remained connected to the ovigerous hairs after hatching. In developing embryos, both protein bands could be stained immunochemically at least 10 days before hatching. But OHSS biological activity appeared only 3 days before hatching. The immunoreactive protein bands were not observed in the zoea, but OHSS bioreactivity was present, though greatly reduced. The 32-kDa protein, at least, is probably an active OHSS, and the 30-kDa protein band may also be OHSS-related. The OHSS appears to be produced and stored by the developing embryo. Upon hatching, most of the material may be trapped by the remnant structures, and the remainder is released into the ambient water.

Introduction

After egg-laying, the embryos of intertidal and estuarine crabs—and indeed most decapod crustaceans—are encased in a thick, protective capsule composed of two or three layers; these capsules are then attached to the female's ovigerous hairs through the funiculus and investment coat (Yonge, 1937, 1946; Cheung, 1966; Goudeau and Lachaise, 1983). The capsule breaks open during or after embryonic development, and hatching occurs (Davis, 1968, 1981; Saigusa, 1997).

The funiculus and investment coat, as well as the broken egg capsule, remain attached to the hairs after hatching (for further details, see Saigusa, 1994). But at the time of hatching, an active factor we call ovigerous-hair stripping substance (OHSS) is released from the embryo and causes these remnant structures to slip off the hairs (Saigusa, 1994). The stripping of these remnant structures is very important because it leaves the hairs clean, unbroken, and thus prepared to incubate the next clutch of embryos (Saigusa, 1995).

The funiculus, the coat that wraps the ovigerous hairs, and the outermost layer of the egg capsule are all composed of the same material (Saigusa *et al.*, unpub. data), and OHSS may be an enzyme that acts on this material. In many other animals, a hatching enzyme is released from the embryos upon hatching and digests the layers of the egg case (*e.g.*, Yamagami, 1988; Lepage and Gache, 1989; Roe and Lennarz, 1990; Helvik *et al.*, 1991). However, there is no evidence, even after electron microscopy studies, that the layers of the egg capsule encasing crab embryos are digested by OHSS (Saigusa *et al.*, unpubl. data). So the notion that OHSS is a crustacean hatching enzyme remains unsubstantiated.

Although the cells that produce OHSS have not yet been identified, we do know that this substance is secreted by the embryo, and not by the female. The molecular mass of OHSS was estimated by primary gel filtration to be 15–20 kDa (Saigusa, 1995), but no other chemical characteristics are known. If we are ever to characterize the physiological mechanism of OHSS, or its expression during development under the control of a circatidal clock (Saigusa, 1992, 1993, 1997), a purified preparation must first be obtained.

Here we describe a relatively simple procedure for purifying OHSS from the hatch water of an estuarine terrestrial crab, *Sesarma haematocheir*, and we also provide a preliminary characterization of this material. A polyclonal antiserum was raised against OHSS and purified so that, by immunochemical staining, we could examine the appearance of OHSS in the developing embryos and its disappearance after hatching. Furthermore, we assessed whether OHSS is still associated with the remnants of the embryonic attachment structures that remain after hatching and larval release.

Materials and Methods

Larval release and collection of hatch water

Specimens of *Sesarma haematocheir*, the estuarine terrestrial crab used in this study, were collected at Kasaoka, Okayama Prefecture, Japan. Here, the thicket inhabited by the crabs is separated from the shore of a tidal creek by a small road (for their habitat, see Saigusa, 1982). Just after sunset, between 1900 and 2000 h, for several days around the time of the full or new moon, large numbers of ovigerous female appear onto this road on their way to the shore. Thus exposed, they can easily be captured.

In 1994, more than 3000 females were captured on the road and placed individually into large plastic containers (10 cm in diameter, 15 cm in height) containing 30 ml of very clean ground water obtained near the collecting site. The females released their larvae into this water. Immediately thereafter, the females were removed from the containers, and the water was filtered through a nylon mesh that retained the larvae. The filtered hatch water was accumulated in 1-liter bottles, transported 50 km to the laboratory, and finally frozen at -20°C .

The method of collection was changed in 1996. In the three years 1996, 1997, and 1998, ovigerous females were still collected at the same site at Kasaoka, but they were first disinfected in ice-cold 70%–80% ethanol for a few minutes, then washed with distilled water, and finally placed individually in the large plastic containers, but without water. These containers were transferred to the laboratory, where each crab was immediately placed in a small, covered plastic cup (5 cm in diameter, 6 or 8 cm in height) containing 9 ml of distilled water. As soon as larvae were released, the zoeas were removed by filtration through nylon mesh,

and the remaining water was then passed again through a filter paper. The resulting hatch water was pooled in a 50-ml plastic bottle and immediately stored at -40°C until used. Most females incubate their next clutch of embryos a few days after larval release. The females were therefore kept in the laboratory for a month, and hatch water was obtained from their second larval release.

Since the OHSS activity of hatch water declines constantly at ambient temperatures, the second method, in which the material is frozen immediately after production, yielded hatch water solutions with higher concentrations of OHSS. Moreover, since all of the female crabs survived and were able to incubate another clutch of embryos, we are confident that the disinfection in ethanol and the dry transport were not deleterious to the animals.

Most of the hatch water used in this study was collected in 1994, but some experiments were carried out with material collected in 1996–1998. The concentrations of OHSS obtained by the two methods would certainly have been different, but the biochemical properties observed were virtually identical.

Bioassay of OHSS activity

Our biological assay of OHSS is based on the ability of chemically fixed ovigerous setae to respond to OHSS. In brief, an ovigerous seta with its attached embryos, all in early stages of development, were excised from a female crab, fixed in 70% ethanol, and then stored in the refrigerator at 4°C until used. Ethanol-fixed setae with their attached clumps of embryos respond well to exogenous OHSS, even after several years of cold storage at 4°C (unpub. data).

Shortly before a bioassay was to be performed, the fixed setae were suspended in distilled water (DW) to wash out the ethanol, and then placed in a glass dish with DW. The tip of each ovigerous seta was cut away, and the remainder was subdivided into four segments under the stereomicroscope (see Saigusa, 1995). Each segment was placed on a paper towel to remove attached water, and one or two (or three in some experiments) of these segments were placed in each of the wells (each 0.8 cm in diameter, 1.7 cm in height) of a plastic culture dish; the wells also contained 300–500 μl of a fraction eluted through chromatography. The culture dish was shaken slowly on a mechanical shaker for 1.0–2.2 h at about 23°C .

Recall that each seta is equipped with 10–15 whorls of ovigerous hairs to which the embryos are actually attached, and that the number of ovigerous hairs is about 10–20 per whorl (see Saigusa, 1994, 1995). For example, each of the three segments of a seta would contain 2–5 whorls (30–100 ovigerous hairs). After the incubation described above, each setal segment with its cluster of embryos was again placed in a glass dish with DW. This dish was put under a

stereomicroscope, and fine forceps were used to pull the embryos gently away from the ovigerous hairs. The percentage of hairs in each whorl that were stripped clean but were still undamaged was calculated. The activity of OHSS was usually taken as the mean of 3–10 whorls (one or two segments); the standard deviation was also calculated.

Purification procedure

Stored, frozen, crude hatch water was thawed and centrifuged at 18,000 rpm for 30 min at 4°C to remove the solid materials. The hatch water was saturated with $(\text{NH}_4)_2\text{SO}_4$ powder, left overnight, and centrifuged at 18,000 rpm for 30 min. The supernatant contained no OHSS activity. The precipitate was dissolved in 100 mM Tris-HCl buffer (pH 9.0) and, at this stage, could also be stored at -20°C. This material was called "concentrated hatch water." OHSS was purified further in three steps, as described below. The procedures were all carried out with a fast protein liquid chromatography system (FPLC; Pharmacia) in an experimental chamber with the temperature controlled at 4°C; protein elution was monitored at 280 nm. OHSS rapidly loses its activity during purification, so the following procedures were completed within 12 h.

Step 1: Hydrophobic chromatography. Concentrated hatch water (described above) was mixed with an equal quantity of 20 mM Tris-HCl buffer (pH 9.0) containing 300 mM Na_2SO_4 . This medium was applied to a column containing 10 ml of HiTrap-Octyl-Sepharose 4FF (prepack column, Pharmacia) equilibrated with 20 mM Tris-HCl buffer containing 300 mM Na_2SO_4 (pH 9.0). The column was eluted with a linear gradient of Na_2SO_4 : -8 mM/min (-2 mM/ml and a flow rate maintained at 4.0 ml/min). Fractions of 10 ml were collected.

Step 2: Ion-exchange chromatography. The active fractions from step 1 were pooled (60 ml), and this sample was applied to an anion-exchange column (MONO-Q HR5/5, prepack, Pharmacia; 0.5 × 5 cm). The column had been pre-equilibrated with 20 mM Tris-HCl buffer (pH 9.0), and the sample was eluted with the same solution. The flow rate was 1.0 ml/min, and 2-ml fractions were collected. The fractions from the void volume were pooled, and were concentrated to 500 μl with an ultrafiltration membrane (YM 10, Amicon).

Step 3: Molecular-sieve chromatography. A sample of these concentrated active fractions (500 μl) was fractionated by molecular-sieve chromatography (gel filtration) (Superdex 75 HR10/30, prepack, Pharmacia). The column had been equilibrated previously with 20 mM Tris-HCl buffer containing 150 mM NaCl (pH 9.0). The sample was eluted with the same buffer at a flow rate of 0.25 ml/min, and 1-ml fractions were collected. The molecular mass of OHSS was determined by comparison with the elution volume of the following marker proteins: glutamate dehydrogenase (55.6

kDa), aldolase (39.2 kDa), trypsin inhibitor (20.1 kDa) (Sigma Chemical Co.).

Effects of temperature and pH

The active fractions from molecular-sieve chromatography (fractions 6–8; Fig. 2A) were pooled and incubated for 15 min at temperatures from 4°C to 100°C, and were immediately returned to 23°C. Segments of the ovigerous seta with their attached embryos were incubated with these solutions for 1 h at 23°C, and OHSS activity was bioassayed. In another experiment, the pooled active fractions were maintained at 4°C and 23°C for 0–120 h. These solutions were then bioassayed for 1 h.

The effects of pH were examined similarly. Buffers used for this study were as follows: 100 mM Na-acetate (pH 3.0 and 5.0); 100 mM Tris-HCl (pH 7.0 and 8.5); and 100 mM glycine-NaOH (pH 10.5). Active fractions were mixed with an equal quantity of each buffer, and the OHSS activity was bioassayed for 1 h.

In each experiment (A–C), assays were repeated three times with the same OHSS solution. The mean percentage of stripped hairs in 2–5 whorls of the hair was first estimated, and the mean of three assays was estimated.

Electrophoretic analysis (SDS-PAGE)

SDS (sodium dodecyl sulfate)-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970). Each fraction from gel filtration was concentrated by passage through an ultrafiltration membrane (Centricon 10, Amicon). The filtered material was dissolved in an equal quantity of lysis buffer [composition: 5% SDS, 5% 2-mercaptoethanol, 8 M urea, 5 mM EDTA (ethylenediaminetetraacetic acid), 5% sucrose, in 125 mM Tris-HCl (pH 6.8)], and then kept at room temperature for 30 min. The molecular mass markers employed were glutamate dehydrogenase (55.6 kDa), aldolase (39.2 kDa), triosephosphase isomerase (26.6 kDa), trypsin inhibitor (20.1 kDa), and lysozyme (14.3 kDa) (Sigma Chemical Co.). The gels (15%) were transblotted onto PVDF (polyvinylidene difluoride) membranes (Clear Blot Membrane P; Atto, Japan), and were stained with Coomassie brilliant blue R-250 (CBB).

Electrophoresis of the active fractions without denaturation (native SDS-PAGE) and bioassay with the crushed gel

The active fractions from gel filtration were pooled (2 ml) and concentrated to less than 200 μl by passage through the ultrafiltration membrane. This material (80 μl) was dissolved in a solution containing 45 mg of saccharose added in 100 μl of 0.5 M Tris-HCl (pH 6.8), and was electrophoresed on an SDS-polyacrylamide gel (15%). After electro-

phoresis, a narrow strip of this gel was cut parallel to the direction of migration, and then stained with CBB. This strip was used to indicate the position of the protein bands. The remainder of the gel was cut into four equal segments perpendicular to the direction of migration. Each of these gel segments was crushed with a pestle, 500 μl of DW was added, and the OHSS activity was bioassayed.

Preparation of polyclonal antiserum, electrophoresis, and western blotting of OHSS

In preliminary experiments, a 32-kDa protein band seen on SDS-PAGE was cut from the gel with a sharp knife, mixed with Freund's complete adjuvant, and injected into a commercial white rabbit. These treatments—four injections, 2 weeks apart—yielded no antiserum when assayed by western blotting. Therefore, the pooled active fractions from gel filtration (2 ml; 6 and 7 in Fig. 2A) were mixed with the adjuvant, and two additional injections, 2 weeks apart, were given to the same rabbit.

In western blotting, the electrophoresed sample was transblotted onto PVDF membrane, and immunoreactivity was detected by chemobioluminescence with ECL western blotting reagents (Amersham). For the primary antibody, the polyclonal antiserum was diluted 1:1000 with 0.5% nonfat milk in T-TBS (10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20). The PVDF membrane was incubated with the primary antibody for 1 h at room temperature, then washed five times with T-TBS. The secondary antibody was peroxidase-conjugated goat anti-rabbit immunoglobulin (Cappel) diluted 1:5000 with 0.5% nonfat milk in T-TBS. After a 1 h incubation with the secondary antibody at room temperature, the membrane was washed five times with T-TBS, and was further incubated (for 1 min at room temperature) with ECL western blotting reagents. This membrane was exposed to Hyperfilm ECL (Amersham).

Affinity purification of the OHSS antiserum

The pooled active fractions from molecular sieve chromatography (6 and 7 in Fig. 2A) were concentrated, electrophoresed on 15% SDS gel, and then transferred to a PVDF membrane. A narrow strip of this membrane was then cut on both edges (5 mm in width), parallel to the direction of migration. These filter strips were stained with CBB and were used to indicate the position of each protein band; the membrane was not stained with CBB, except these strips.

Two protein bands located with the CBB-stained filter strips, an upper (32 kDa) and lower (30 kDa), were cut separately from the membrane. Each band was further cut into pieces and was incubated for 24 h in 1 ml antiserum at 4°C. The pieces of membrane were then rinsed in PBS (pH 7.4) for 10 min, and this operation was repeated 5–6 times. The membrane pieces were overlain with 200 μl of 0.2 M

glycine-HCl (pH 2.0) for 10 min, on ice, to elute bound antibody. The eluate was adjusted to pH 7.4–7.5 by the addition of 30–32 μl of 1 M Tris.

For staining, a 1:1000 dilution of the antibody that was eluted from either the 32-kDa or 30-kDa band was used as the primary; dilution was with 0.5% nonfat milk in T-TBS. The secondary antibody was peroxidase-conjugated goat-rabbit immunoglobulin (Cappel) diluted 1:5000 with 0.5% nonfat milk in T-TBS.

Because the two bands were at most 2 mm apart on the gel and were not stained with CBB, we could not be sure that the two proteins had been completely separated. Therefore, concentrated active fractions from molecular-sieve chromatography (6 and 7 in Fig. 2A) were electrophoresed on 15% SDS gel for a longer period (9 h), and were then transferred to a PVDF membrane. This procedure produced a gap of 5–6 mm between the upper and lower bands. The whole membrane was stained with 0.01% Ponceau S (Sigma) in 5% acetic acid for 10 min. The protein bands, 2 mm wide, were cut out and the middle strip (about 2 mm wide) was excluded. The bound antibodies were then eluted, as described above.

Immunochemical detection of OHSS during embryonic development

At various times before hatching, an embryo cluster with its attached hairs and seta (*i.e.*, one-third of an ovigerous seta altogether) was detached from a single female, crushed with a pestle in distilled water (300 μl) for 5 min, and denatured by the addition of 300 μl of the lysis buffer (pH 6.8). The solution was centrifuged at 15,000 rpm for 20 min. Fifty microliters of the supernatant was pipetted, and 100 μl of DW and 100 μl of the lysis buffer were added to this supernatant. Thirty microliters of this solution was subjected to electrophoresis, and OHSS was examined by the immunochemical staining of western blots. The purified antibody was used in the experiments.

OHSS was also examined as follows. Embryo clusters were crushed with a pestle in 500 μl of DW, and immediately centrifuged for 20 min. Then the supernatant was denatured in the lysis buffer described above for 1 h, electrophoresed, and the OHSS was examined by the immunochemical staining of western blots. The sediment was denatured separately by the addition of the lysis buffer for 1 h. The solution was further centrifuged for 20 min, and the supernatant was subjected to electrophoresis followed by immunochemical detection.

Appearance of OHSS activity in developing embryos: bioassay with crushed embryos

One-third of the embryo clusters attached to an ovigerous seta were detached from a single female every day until hatching, crushed in 500 μl of DW, and then centrifuged at

15,000 rpm. The OHSS activity contained in these samples (*i.e.*, the supernatant and sediment of crushed embryos) was bioassayed.

Bioassay of OHSS activity in broken egg cases

As described elsewhere (Saigusa, 1994), remnants of the embryonic attachment system (*i.e.*, broken egg cases, funiculi, and the coat investing ovigerous hairs) remain on the ovigerous hairs after hatching and larval release. These remnants were removed with fine forceps after hatching, and stored at -20°C until used. Ovigerous hairs and setae were not contained in these remnants. So the possibility that the OHSS activity is present in the ovigerous hairs and setae of the female was excluded.

Five hundred milligrams of the remnant matter (wet weight) was thawed and washed repeatedly with DW, and centrifuged for 20 min (15,000 rpm). The sediment was crushed in 600 μl of DW; the suspension was divided into two wells of a plastic dish (each 300 μl), and OHSS activity was bioassayed for 1.5 h and 2.0 h.

We also determined whether the OHSS activity could be

extracted with the detergent (Triton-X). Remnant matter (500 mg wet weight) was thawed and washed repeatedly with DW. The remnants were then crushed in 600 μl of Triton-X solution dissolved in DW (5%, 10%, and 20%), and were held for 30 min at room temperature (23°C). Each suspension was centrifuged for 20 min (15,000 rpm), the supernatant was divided into two wells of a plastic culture dish (each 300 μl), and OHSS activity was bioassayed for 1.5 and 2.2 h.

Results

Chromatography and estimation of the molecular mass

Crude hatch water from about 90 females was saturated with $(\text{NH}_4)_2\text{SO}_4$ overnight; no OHSS activity was found in the supernatant. The precipitate was dissolved in 100 mM Tris-HCl buffer. This solution (concentrated hatch water) was subjected to hydrophobic chromatography, and each fraction (10 ml) was bioassayed for 1.5 h.

As shown in Figure 1, most proteins were removed by this fractionation. The peak of OHSS activity was very

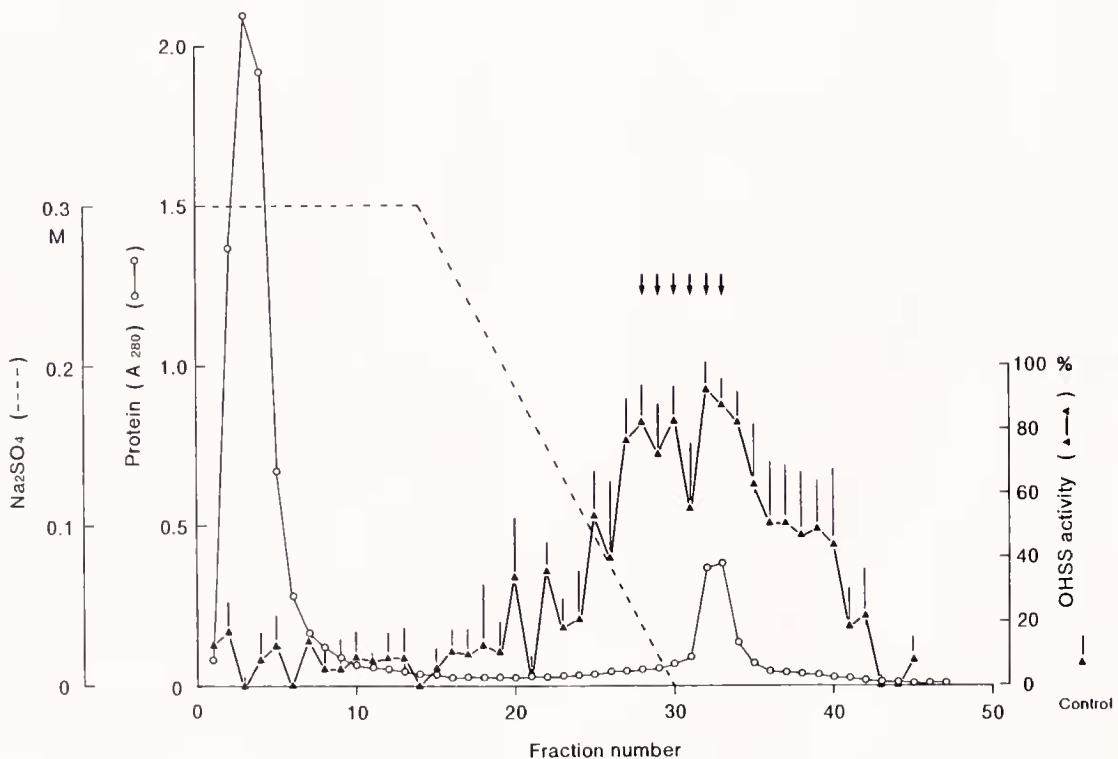


Figure 1. Elution of OHSS activity from a hydrophobic column (HiTrap-Octyl-Sepharose 4FF; Pharmacia). Concentrated hatch water from 90 females was applied to a column previously equilibrated with 20 mM Tris-HCl buffer containing 300 mM Na_2SO_4 , and the proteins were eluted with a linear gradient: $-8 \text{ mM } \text{Na}_2\text{SO}_4/\text{min}$ (broken line). Open circles (○); 280 nm absorption indicating protein concentration in each fraction (10 ml). Bioassay: incubation time, 1.5 h; OHSS activity, percentage of stripped, unbroken ovigerous hairs per whorl; data points are the mean percentages of 3–10 whorls (▲ solid triangles); vertical bars indicate standard deviation; control assay (▲ bottom right), incubation with buffer (details of assay in Methods). Vertical arrows indicate the 6 active fractions (60 ml) applied to anion exchange chromatography.

broad, and only half of it coincided with a protein peak that eluted after 0 mM Na₂SO₄ was reached. A bioassay with an incubation of 1.0 h was also attempted; the results were similar to those shown in Figure 1, although the peak of activity was narrower than that with the longer incubation (not shown).

The pooled active fractions from hydrophobic chromatography (60 ml; six vertical arrows in Fig. 1) were subjected to anion exchange chromatography. The OHSS activity appeared in the pass-through fractions (not shown).

These active fractions (60 ml) were concentrated to 500 μ l by ultrafiltration and were then subjected to molecular-sieve chromatography. Each fraction was bioassayed for 1.5 h. As shown in Figure 2A, the activity appeared as a single peak in fractions 6–8. The molecular mass of the eluted protein peak was estimated at 35 kDa by comparison with standard proteins. Similar results were also obtained with the solutions bioassayed for 1.0 h (not shown).

SDS-PAGE

Each fraction eluted in molecular-sieve chromatography (Fig. 2A) was concentrated, and the proteins were analyzed by SDS-PAGE. As shown in Figure 2B, two common protein bands appeared in fractions 6 and 7, both of which had high OHSS activity (Fig. 2A). Fraction 8, which also had high OHSS activity, had little or no staining. The molecular masses of these bands were estimated to be 32 kDa and 30 kDa by comparison with the marker proteins. An additional band (22 kDa) appeared in fractions 5 and 6; it is very weak in Figure 2B, but is clear in Figure 5A. It was clear that the OHSS activity bioassayed with ovigerous setal segments does not correspond to the 22-kDa band in fractions 5 and 6 (compare Fig. 2A with Fig. 2B).

The concentrated active fractions (6 and 7) from molecular-sieve chromatography were also electrophoresed without denaturation; the proteins were clearly separated (Fig. 3A). The gel was cut into quarters, and each segment of the gel was bioassayed for 1.5 h and 2.0 h. The OHSS activity appeared in the second segment (b) of the gel strip (molecular mass between 40 kDa and 23 kDa), which contained the 32-kDa and 30-kDa protein bands (Fig. 3B).

Characterization

Active fractions 6–8 from molecular-sieve chromatography (Fig. 2A) were pooled, and the thermostability of the OHSS solution was examined. Active solutions that were exposed for 15 min to temperatures between 4°C and 80°C showed virtually no decrease in OHSS activity. In contrast, solutions exposed to 100°C lost activity (Fig. 4A).

Least-square regression lines fitted to the data (Fig. 4B) indicated that active solutions incubated at either 4°C or room temperature (about 23°C) for up to 70 h showed no significant decrease in activity, and that there was no sig-

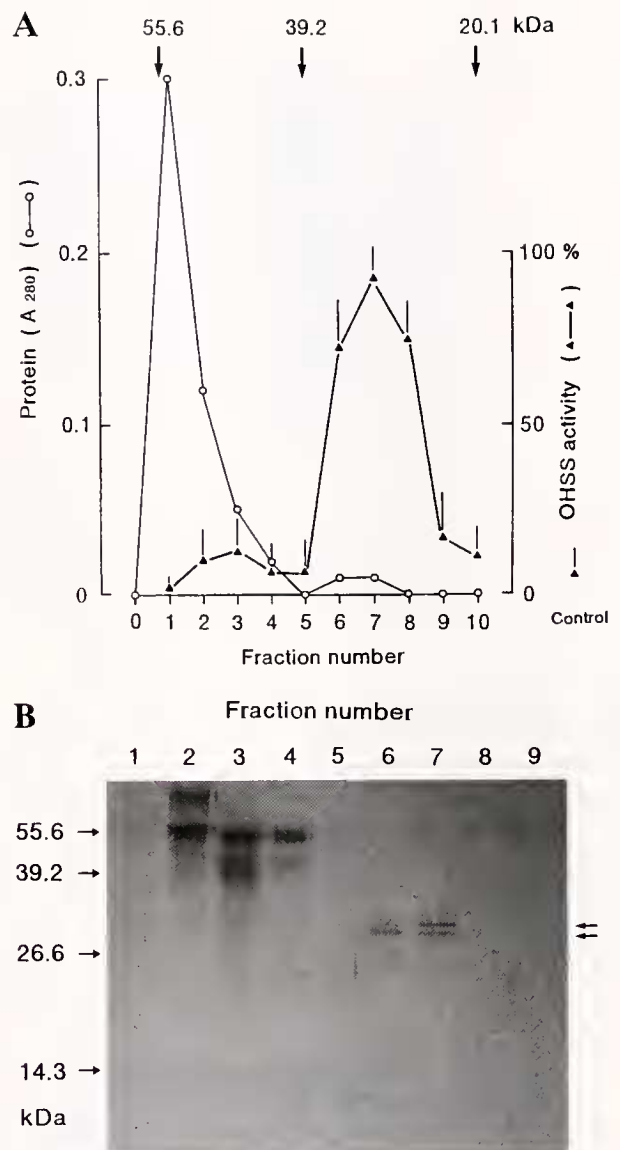


Figure 2. Purification of OHSS activity by molecular-sieve chromatography with protein analysis on SDS-PAGE. (A) The pass-through fractions (60 ml) from anion-exchange chromatography were concentrated by ultrafiltration, and an aliquot (500 μ l) was applied to molecular-sieve chromatography; fractions are 1 ml. Open circles (○): protein concentration in each fraction (280 nm absorption). Solid triangles (▲): the OHSS activity of each fraction; bioassays carried out for 1.5 h with one (or two) ovigerous setal segments per fraction; error bars: standard deviation. Control assay (▲ bottom right). Downward-pointing arrows indicate the molecular masses of marker proteins: glutamate dehydrogenase (55.6 kDa), aldolase (39.2 kDa), and trypsin inhibitor (20.1 kDa). (B) Analysis, by SDS-PAGE, of the proteins in each fraction (1–9) eluted from molecular-sieve chromatography. The polyacrylamide gel was transblotted to a PVDF membrane which was stained with Coomassie brilliant blue. The marker proteins were glutamate dehydrogenase (55.6 kDa), aldolase (39.2 kDa), triosephosphate isomerase (26.6 kDa), and lysozyme (14.3 kDa). The two bands that appear in fractions 6 and 7 (arrows to the right) have molecular masses of 32 kDa and 30 kDa.

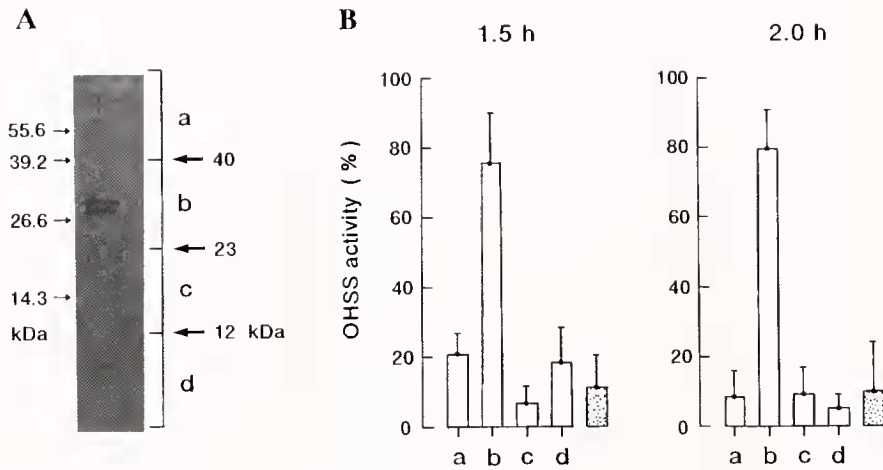


Figure 3. Distribution of OHSS activity in a polyacrylamide gel divided into four equal segments after SDS-PAGE. (A) The gel. Numbers at the left of the small arrows indicate the molecular masses of the standards (see Fig. 2B). The molecular mass limits of each gel segment are indicated by the large arrows. (B) Bioassay with two or three ovigerous setal segments. Open bars indicate OHSS activity of each gel segment. Incubation periods for the bioassay: 1.5 h and 2.0 h. Stippled bars: controls; the ovigerous setal segments were incubated in DW for 1.5 h and 2.0 h. Error bars: standard deviation.

nificant difference between the two experiments. Solutions maintained for longer times (100 and 120 h) decreased in activity in both experiments.

Figure 4C shows the pH dependency of OHSS activity. After an assay incubation of 1 h, the pH optimum was quite broad, about 7.0–11.0.

Specificity of antibodies and affinity purification

An antiserum raised from molecular-sieve chromatography (fractions 6 and 7) detected only two strong protein

bands on SDS-PAGE, and they appeared in fractions 6–8 (Fig. 5). These immunostained protein bands clearly corresponded to the peak of OHSS activity in Figure 2A.

The antibodies that had stained the 32-kDa and 30-kDa protein bands were purified by immunochemical affinity to determine the specificity of binding. As shown in Figure 6A, the antibodies eluted from the 32-kDa protein band on SDS-PAGE stained the 30-kDa protein as well; and the antibodies eluted from the 30-kDa band similarly stained both the 30-kDa and 32-kDa proteins.

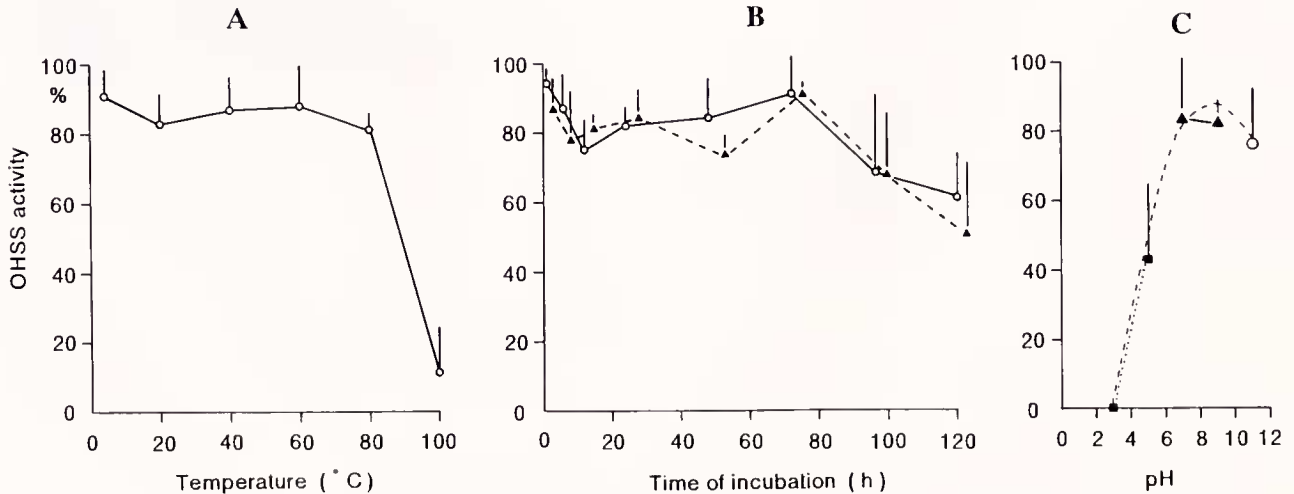


Figure 4. Characteristics of OHSS activity. (A) Heat stability. Active solutions were incubated for 15 min at each temperature, and OHSS activity was bioassayed for 1 h. (B) Effects of prolonged incubation (up to 5 days). For each time, active solutions were maintained at either 4°C (○) or 23°C (▲), and the activity was bioassayed for 1 h. (C) Dependence of activity on pH. Buffers: 100 mM Na-acetate (■); 100 mM Tris-HCl (▲); 100 mM Gly-NaOH (○). The activity was bioassayed for 1 h. Broken line in (C) indicates the least-square regression curve fitted to the data. Error bars: standard deviation of three experiments.

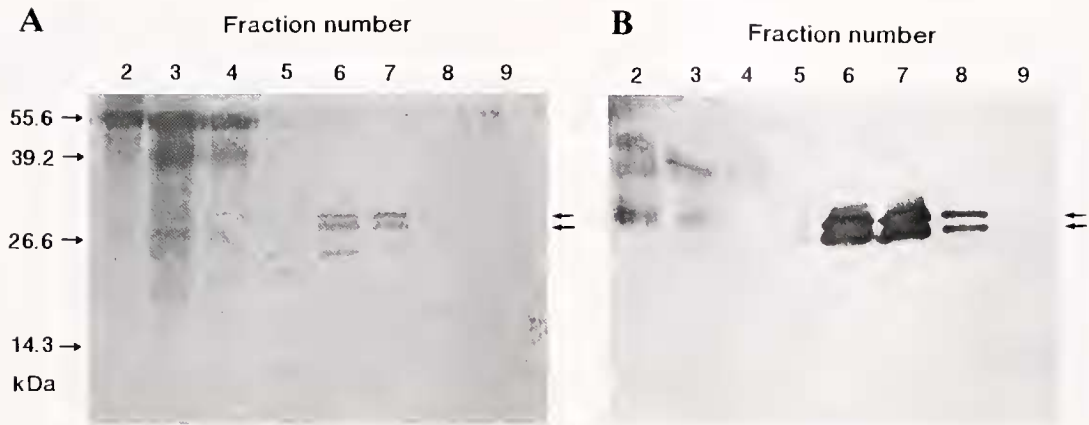


Figure 5. Specificity of the polyclonal antiserum raised against OHSS. (A) SDS-PAGE of fractions eluted from molecular-sieve chromatography. The polyacrylamide gel was transblotted onto a PVDF membrane and stained with Coomassie brilliant blue. (B) Immunostaining of the PVDF membrane with a polyclonal antiserum raised to OHSS. Numbers to the left show the molecular masses of the standards shown in Figure 2B. The intensely immunostained bands in fractions 6–8 have masses of about 32 kDa and 30 kDa (arrows to the right).

In this experiment (Fig. 6A), however, the 32-kDa band was at most 2 mm apart from the 30 kDa band on the gel. In addition, the PVDF membrane was not stained with CBB, except the narrow strip that had been cut on both sides (5 mm in width), because the antibodies were not bound to CBB-stained proteins. So these protein bands could have been incompletely separated.

In Figure 6B, concentrated active fractions from molec-

ular sieve chromatography (6 and 7 in Fig. 2A) were electrophoresed for a longer period (9 h), which produced a gap of 5–6 mm between the upper and lower bands. The whole membrane was stained by Ponceau S, and each protein band (2 mm in width) was subjected to affinity purification. Again, the antibody raised from the 32-kDa band bound to the 30-kDa band as well; and the antibody produced from the 30-kDa band also recognized the 32-kDa band (Fig. 6B).

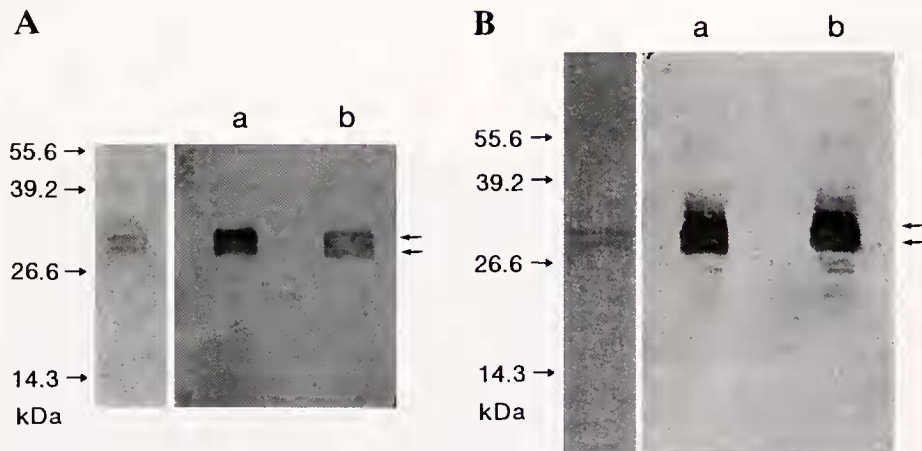


Figure 6. Immunostaining with antibodies affinity purified from SDS-PAGE bands that had OHSS activity. SDS-PAGE of the proteins from molecular-sieve fractions 6 and 7 (see Fig. 2A) was carried out for either 3.5 h (A) or 9 h (B), the longer electrophoresis producing the greater separation between the active bands at 32 kDa and 30 kDa. The bands were cut out of the PVDF membranes, incubated with a polyclonal antiserum raised to OHSS, and the bound antibodies were eluted (see Methods). These antibodies, obtained from the short and long electrophoresis, were applied to two new SDS-PAGE runs, both of 3.5 h; these final runs are shown in this figure. Immunostaining was effected by antibodies bound (a) to protein in the upper band (32 kDa), and (b) to the protein in the lower band (30 kDa). The strips to the left of the two panels were stained with Coomassie brilliant blue (A) and Ponceau S (B); the numbers (with arrow) are the molecular masses of the standard molecules (identified in Fig. 2B).

OHSS activity in the post-hatching remnants of the embryo attachment system

After hatching, the broken egg cases, funiculus, and investment coat remain attached to the ovigerous hairs. The female picks these remnant attachment structures off the ovigerous hairs, but this must occur after the OHSS released with the hatch water has been greatly diluted in the estuary. We therefore examined the possibility that at least some OHSS is present in the remnant attachment structures themselves.

The remnants were collected and stained with FITC-conjugated, OHSS antiserum (Fig. 7). OHSS was clearly detected all over the remnants, including the prezoal cuticles. In contrast, the egg capsule, funiculus, and investment coat did not react to the FITC-conjugated OHSS antiserum when the embryos were squeezed in the egg cases (not shown).

When samples for electrophoresis and immunoblotting were prepared from remnants that had been crushed and denatured, only the 32-kDa band was detected (Fig. 7, right).

Remnants stored at -20°C were thawed and crushed, and suspensions of this material (not centrifuged) were bioassayed for 1.5 h and 2.0 h. As shown in Table I, strong OHSS was detected in this solution. In another experiment, the remnants were thawed and crushed, and then treated with 5%, 10%, and 20% Triton-X. After centrifugation for 20 min (15,000 rpm), the supernatant was bioassayed for 1.5 h and 2.2 h and showed strong OHSS activity, particularly at the longer incubation (Table II).

Appearance of OHSS in the developing embryos

Because OHSS immunoreactivity and biological activity occur in the remnants after hatching, we determined when,

during development, these activities would appear. Immunochemical and biological observations were made.

Immunochemistry. Embryo clusters (one-third of an ovigerous seta) were taken at successive times from a single female and crushed; the suspensions were denatured with the lysis buffer *before* centrifugation. As shown in Figure 8, the 32- and 30-kDa proteins became noticeable at least 2 weeks before hatching. The 30-kDa band was the stronger of the two bands at 10 and 6 days before hatching; but its intensity declined and became very faint in embryos 4 h before hatching; and it disappeared completely in post-hatched zoeas. In contrast, the 32-kDa band appeared later than the 30-kDa band, but it was still quite clear in embryos 4 h before hatching, and again was not detected in the post-hatched zoeas. In addition to the two bands of OHSS-related protein, an immunoreactive band appeared at about 55 kDa, from 14 to about 2 days before hatching (Fig. 8).

In another female, the supernatant and the sediment were denatured separately by the lysis buffer *after* the embryo clusters had been crushed and centrifuged. In the supernatant (Fig. 9A), OHSS first appeared as a weak 32-kDa band 14 days before hatching. The 30-kDa band was noticeable 10 days before hatching, and the amount increased abruptly in the embryos collected 6 days before. This protein decreased markedly just before hatching, but was still visible 4 h before hatching.

On the other hand, the sediment contained broken egg cases, funiculi, the investment coat, ovigerous hairs, prezoal cuticles, and probably only a portion of the embryos. When this material was centrifuged and the supernatant was examined on the same female (Fig. 9B), the 30-kDa band appeared weakly in the lanes derived from embryos collected 10, 6, and 4 days before hatching; but it was absent at other times. In contrast, the 32-kDa band

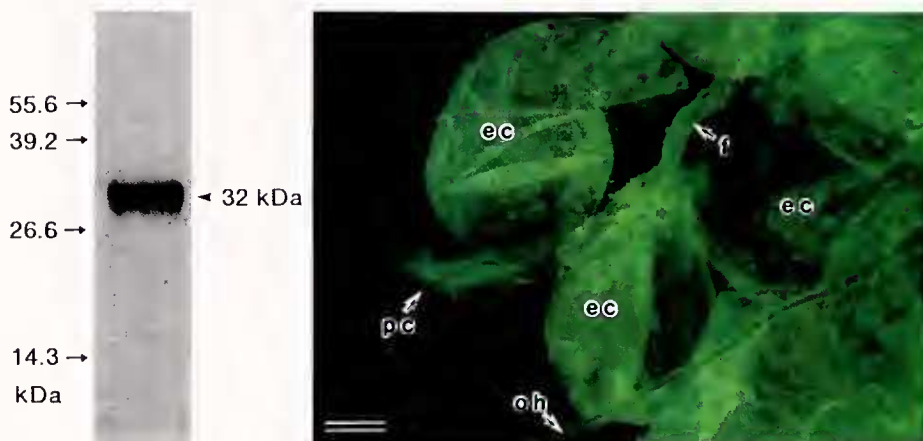


Figure 7. Immunochemical staining of the structures remaining attached to a female's ovigerous hairs after hatching. Left: the immunoblot of an extract of the remnants subjected to SDS-PAGE. Arrowhead: 32-kDa protein band. Numbers to the left show the molecular masses of the standard molecules (as in Fig. 2B). Right: the remnants stained with polyclonal FITC-conjugated OHSS antiserum. *ec*: broken egg case; *f*: funiculus; *pc*: prezoal cuticle; *oh*: female ovigerous hair (see fig. 2 in Saigusa, 1994). Scale: 100 μm .

Table I*Bioassay of suspensions of crushed attachment structures remaining after hatching*

	Incubation*	
	1.5 h	2.0 h
Control†	1.2 ± 2.4%(5)	3.8 ± 6.3%(6)
Experiment	81.1 ± 7.4%(6)	93.5 ± 4.3%(4)

* Values are the mean percentage of unbroken, stripped ovigerous hairs on each whorl; number of whorls on the subdivided segments of the ovigerous seta is given in parentheses.

† Incubation of embryo clusters in distilled water.

became very distinct 6 days before hatching, and was virtually the only band that appeared from 2 days before, until hatching. A 55-kDa band was also detected in the sediment, but was less clear than in the supernatant (Fig. 9A).

Bioassay. Embryo clusters (one-third of an ovigerous seta) were detached from a single female, and were frozen at -20°C . The embryo clusters were thawed and crushed in distilled water, centrifuged, and the OHSS activity in the supernatant was bioassayed for 1.5 h and 2.2 h (Fig. 10A). The OHSS activity began to appear 3 days before hatching, became very strong 6 h before hatching, and was markedly decreased in the zoeas.

The sediment of the embryo clusters was also examined (Fig. 10B). The OHSS activity was again detectable 3 days before hatching; the activity increased near hatching and, like the supernatant, was very strong 6 h before hatching. The zoeas, again, had weak OHSS activity.

Discussion

Ovigerous-hair stripping substance (OHSS) from an estuarine terrestrial crab was purified through three steps of chromatography (Fig. 2A). The activity eluted as a single peak on molecular-sieve chromatography, but still showed two protein bands at 32 kDa and 30 kDa on SDS-PAGE (Fig. 2B). Affinity purified antibodies raised to the active fractions (6 and 7 in Fig. 2A) also bound to two bands at 32 kDa and 30 kDa, corresponding to the bands shown in Figure 2B. Furthermore, immunochemical staining indicated that OHSS-related 32-kDa and 30-kDa bands appear in developing embryos at least 10 days before hatching. This time course, however, did not correspond to the appearance of OHSS activity as bioassayed with ovigerous setal segments. These features invite the following four issues for discussion.

Stability of OHSS

As shown in Figure 4B, purified OHSS retains its activity for at least 80 h, even at room temperature. When crude or

concentrated hatch water was frozen to temperatures lower than -20°C , OHSS was stable. But instability appeared after thawing. For example, active fractions that were eluted from the hydrophobic column lost most of their activity within 12 h (unpub. data).

Crude hatch water contains multiple proteases, and these proteases digest casein (Katsube *et al.*, 1999; see also Saigusa, 1996), and they are not excluded by the hydrophobic chromatography shown in Figure 1. So the disappearance of OHSS activity could be due to its digestion by the caseinolytic proteases that still remain in active solutions.

These caseinolytic proteases do, however, bind to an anion exchange column (MONO-Q) and are therefore excluded with this chromatography. Thus, caseinolytic proteases are not present in the materials applied to the column for molecular-sieve chromatography (Fig. 2A). Consequently, purified OHSS retains its activity for a long period even at room temperature (Fig. 4B).

Evidence that the 32-kDa band is active OHSS

As shown in Figure 2A, OHSS activity always appeared in fractions 6–8 on molecular-sieve chromatography. On SDS-PAGE, 32-kDa and 30-kDa protein bands appeared very clearly in these fractions (Fig. 2B). The second segment (b) of the gel strip containing these two bands certainly showed OHSS activity (Fig. 3). An additional band (22 kDa) appeared in fractions 5 and 6 (Figs. 2B and 5A), but did not correspond to the OHSS activity bioassayed with ovigerous setal segments (compare Fig. 2A with Fig. 2B). Thus, we might speculate that OHSS consists of either or both of the two protein bands at 32 kDa and 30 kDa.

Antibodies raised from fractions 6 and 7 stained the 32-kDa and 30-kDa protein bands (Fig. 5B). These bands also appeared in fraction 8, indicating a good correspondence with the bioassayed OHSS activity (Fig. 2A). OHSS is clearly detected all over the remnants that remain on the ovigerous hairs after hatching (Fig. 7). However, only the

Table II*Bioassay with the extract from the remnants with the detergent (Triton-X)*

	Incubation*	
	1.5 h	2.2 h
Control†	3.6 ± 8.7%(7)	5.8 ± 7.9%(5)
5% Triton-X	27.5 ± 9.3%(4)	92.0 ± 5.7%(3)
10% Triton-X	72.5 ± 15.1%(3)	91.0 ± 2.8%(3)
20% Triton-X	88.3 ± 7.3%(3)	98.5 ± 2.6%(4)

* Values are the mean percentage of unbroken, stripped ovigerous hairs on each whorl; number of whorls on the subdivided segments of ovigerous seta is given in parentheses.

† Incubation of embryo clusters in distilled water.

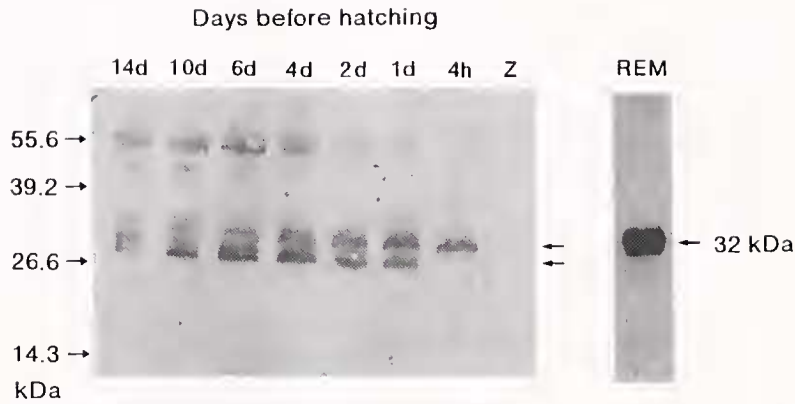


Figure 8. The appearance of immunoreactive OHSS in developing crab embryos. Embryo clusters (one-third of an ovigerous seta) were detached from a single female, crushed, and then denatured with the lysis buffer. The extracts (supernatant) were subjected to SDS-PAGE, and blots were stained immunochemically with the polyclonal OHSS antiserum. *d* (or *h*): days (or hours) before hatching. Z: post-hatched embryos (zoeas) released from the same female. REM: remnant structures remaining attached to the female's ovigerous hairs after hatching. Numbers to the left are the molecular masses of the same markers shown in Figure 2B. Note that only the 32-kDa band is detected in the remnant structures (see Fig. 7).

32-kDa protein band was detected in these remnants (Fig. 8). Furthermore, a strong OHSS activity was also detected in these remnants (Table I), as well as in the supernatant after treatment with detergent (Table II). Hence, we speculate that the 32-kDa band is an active OHSS.

On the other hand, we cannot yet hypothesize that the 30-kDa protein band also has OHSS activity. Since purified antibodies recognized both the 32-kDa and 30-kDa bands (Figs. 6A and 6B), we suppose that these two proteins have very similar sequences; *i.e.*, the bands detected by immunochemical staining are probably both OHSS-related proteins.

The OHSS antiserum detected not only 32-kDa and 30-kDa proteins, but also a band at 55 kDa (Figs. 8 and 9).

When the active fractions 6 and 7 of molecular-sieve chromatography were studied (Fig. 3A), this band was not detected at all. In contrast, when the crushed embryos (Figs. 8 and 9) were examined, the 55-kDa band often appeared clearly; but it did not appear in the remnant (Fig. 7). The 55-kDa band also appeared on molecular-sieve chromatography (Fig. 2B), but it did not have OHSS activity (Fig. 2A). This band also did not appear in any fraction (1–8) of immunostained PVDF membranes (Fig. 5B). So the 55-kDa band in Figure 2B might be different from that appearing in Figures 8 and 9.

As shown in Figure 8, the 55-kDa protein appeared clearly between 14 days and 4 days before hatching; and it became very faint after 2 days before hatching, Figure 9A

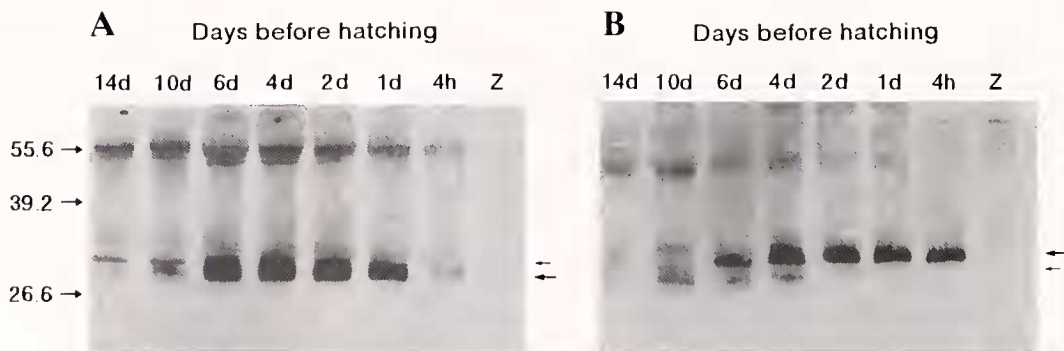


Figure 9. Appearance of immunoreactive OHSS in developing crab embryos. Embryo clusters (one-third of an ovigerous seta) were detached from a single female, crushed, and then centrifuged. The supernatant and precipitate were separately denatured with lysis buffer after centrifugation. (A) The supernatant subjected to SDS-PAGE, and the blots immunostained. (B) Extracts of precipitate of the crushed embryos run on SDS-PAGE and immunostained. *d* (or *h*): days (or hours) before hatching. Z: post-hatched embryos (zoeas) released from the same female. Arrows indicate immunostained 32-kDa and 30-kDa bands. Numbers to the left are the molecular masses of the same markers as in Figure 2B.

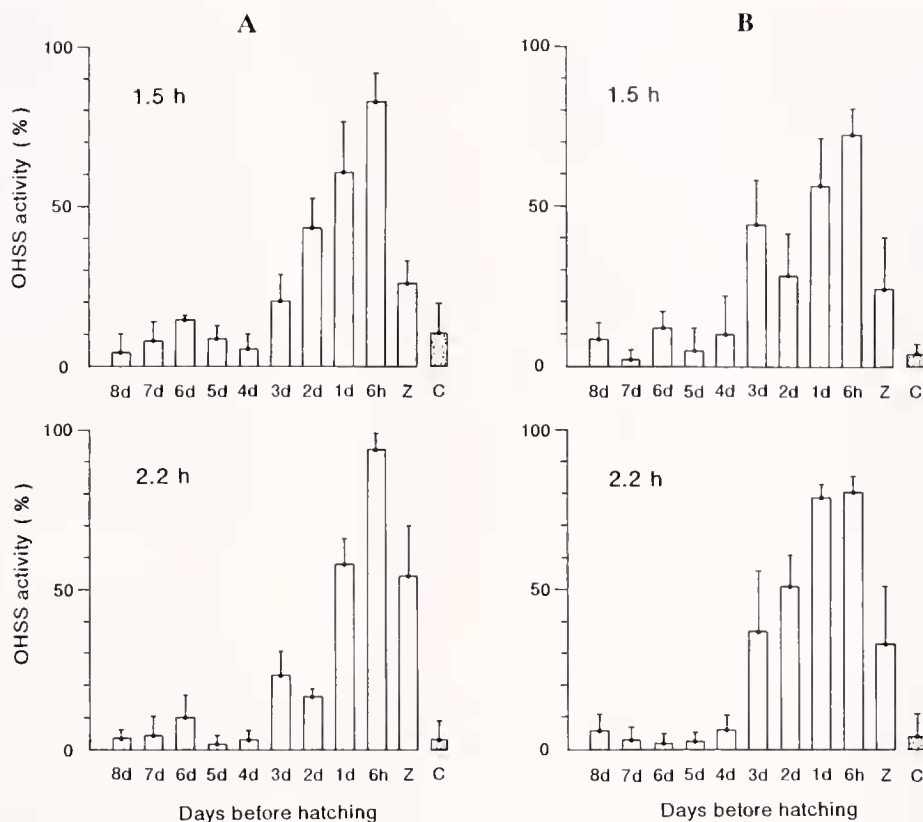


Figure 10. Appearance of OHSS activity in developing crab embryos. Every day, an embryo cluster attached to one-third of an ovigerous seta was detached from a single female. These embryos were crushed, centrifuged, and then the OHSS activity was bioassayed with one or two ovigerous setal segments (open bars). A: Bioassay of the supernatant for 1.5 h (upper panel) and for 2.2 h (lower panel). B: Bioassay of the sediment for 1.5 h (upper panel) and 2.2 h (lower panel). C: Control (incubation with DW; stippled bar). Error bars: standard deviation.

also shows that this protein appears 2 weeks before hatching, and that it stains intensely until 1 day before hatching. These results favor the notion that the 55-kDa protein is, perhaps, a precursor to the 32 and 30 kDa forms.

Behavior of OHSS before and at hatching

When the ovigerous hairs with their attached embryo clusters are gently pulled by a forceps, they are easily broken until the time of hatching. Just after hatching, however, the hairs easily slip out of the coat without damage (see fig. 6 in Saigusa, 1995). Nevertheless, if embryos are crushed before hatching, and the extract is bioassayed, OHSS activity is shown to be present (Fig. 10A and 10B).

The egg capsule, funiculus, and the coat investing ovigerous hairs are not stained by FITC-conjugated OHSS antiserum at least 4 h before hatching (unpub. data). On the other hand, as shown in Tables I and II, remnant structures that are still attached to the ovigerous hairs after hatching show a strong OHSS activity, and

immunoreactive OHSS is associated with the remnants (Fig. 7). We speculate that OHSS is stored somewhere in the embryos as they approach hatching, and that it is released outside the egg capsule when hatching occurs or the embryos are crushed. This OHSS would be strongly attached to broken egg capsules, funiculus, and investment coat, which would be stained by FITC-conjugated OHSS antiserum. As shown in Figure 7, a large amount of immunoreactivity was detected in the broken egg cases. We speculate that most of the OHSS is trapped by the egg capsules upon hatching, and the remainder is released into the ambient water.

With immunochemical staining, the 32-kDa band already appears as a faint band between 14 days (Fig. 9A) and 10 days (Fig. 9B) before hatching. However, bioassay of OHSS (Fig. 10A and 10B) shows the OHSS activity appearing no earlier than 3 days before hatching. Moreover, neither the 32-kDa nor 30-kDa band was detected in post-hatched larvae upon immunochemical staining (Fig. 9A and 9B), but at least some OHSS

activity was detected by bioassay (Fig. 10A and 10B). We cannot fully explain this discrepancy. As described above, OHSS could be stored somewhere in the embryo after it has been produced; at first it may be in an inactive form, being activated a few days before hatching.

Active substances in other species

Morphologically, the coat that wraps the ovigerous hairs, the funiculus, and the outermost layer (E1) of the egg case are all composed of the same material (Saigusa *et al.*, unpub. data). OHSS is likely to be an enzyme that acts on this material, but electron microscopical observations suggest that OHSS does not digest any portion of the egg case (unpub. data). OHSS is neither a collagenase nor a chitinase. If the main component of the embryo attachment structures were glycoproteins, OHSS might act by partially digesting the sugar, and thus softening the coat wrapping the ovigerous hairs.

Since the layers forming the egg case are not digested by OHSS, the notion that this substance is a crustacean hatching enzyme remains unsubstantiated. However, OHSS can be compared with active substances that are released to the outside by the embryos of other species, upon hatching.

Medaka hatching enzyme, which digests a thick inner layer of the egg case, consists of two kinds of proteases: a high choriolytic enzyme (HCE) and a low choriolytic enzyme (LCE) (Yasumasu *et al.*, 1989a, b). The molecular masses of these enzymes are similar—24.0 kDa for HCE and 25.5 kDa for LCE, as determined by SDS-PAGE—but their actions on the egg case are different. That is, HCE digests the inner layer, causing it to swell markedly, and LCE efficiently digests this swollen inner layer; but acting alone, LCE has no effect on this structure. These are distinct enzymes, and they are not interconvertible (Yasumasu *et al.*, 1989a, b; Yasumasu *et al.*, 1992).

Hatching enzymes in sea urchin embryos are known to be released first as a large molecular mass, and then converted into a smaller mass. For example, the hatching enzyme of *Paracentrotus lividus*, purified by Lepage and Gache (1989), is a 57-kDa glycoprotein, highly active on the fertilization envelope. But this enzyme is soon converted into a 30-kDa form with a reduced proteolytic activity on dimethylcasin (about 80%) and with no activity on the fertilization envelope. The reduced molecular size and the loss of activity were hypothesized to be due to autolysis.

Similar results have been reported for *Strongylocentrotus purpuratus* embryos (Roe and Lennarz, 1990); *i.e.*, the hatching enzyme is secreted from the embryos as a 57-kDa form on SDS-PAGE, but it is converted to a 33-kDa form during purification. This conversion was speculated to be

due either to autolysis or to proteolysis by a different protease contained in the sample solution.

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Literature Cited

- Cheung, T. S. 1966.** The development of egg-membranes and egg attachment in the shore crab, *Carcinus maenas*, and some related decapods. *J. Mar. Biol. Assoc. UK* **46**: 373–400.
- Davis, C. C. 1968.** Mechanisms of hatching in aquatic invertebrate eggs. *Oceanogr. Mar. Biol. Annu. Rev.* **6**: 325–376.
- Davis, C. C. 1981.** Mechanisms of hatching in aquatic invertebrate eggs. II. *Oceanogr. Mar. Biol. Annu. Rev.* **19**: 95–123.
- Goudeau, M., and F. Lachaise. 1983.** Structure of the egg funiculus and deposition of embryonic envelopes in a crab. *Tissue Cell* **15**: 47–62.
- Helvik, J. V., D. O. Oppen-Bernsten, and B. T. Walther. 1991.** The hatching mechanism in Atlantic halibut (*Hippoglossus hippoglossus*). *Int. J. Dev. Biol.* **35**: 9–16.
- Katsube, Y., M. Terajima, and M. Saigusa. 1999.** Caseinolytic protease in crab hatch water: preliminary studies of purification, characterization, and its putative role. *Proc. Arthropod. Embryol. Soc. Jpn.* **34**: 1–9.
- Laemmli, U. K. 1970.** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.
- Lepage, T., and C. Gache. 1989.** Purification and characterization of the sea urchin embryos hatching enzyme. *J. Biol. Chem.* **264**: 4787–4793.
- Roe, J. L., and W. J. Lennarz. 1990.** Biosynthesis and secretion of the hatching enzyme during sea urchin embryogenesis. *J. Biol. Chem.* **265**: 8704–8711.
- Saigusa, M. 1982.** Larval release rhythm coinciding with solar day and tidal cycles in the terrestrial crab *Sesarma*. *Biol. Bull.* **162**: 371–386.
- Saigusa, M. 1992.** Control of hatching in an estuarine terrestrial crab. I. Hatching of embryos detached from the female and emergence of mature larvae. *Biol. Bull.* **183**: 401–408.
- Saigusa, M. 1993.** Control of hatching in an estuarine terrestrial crab. II. Exchange of a cluster of embryos between two females. *Biol. Bull.* **184**: 186–202.
- Saigusa, M. 1994.** A substance inducing the loss of premature embryos from ovigerous crabs. *Biol. Bull.* **186**: 81–89.
- Saigusa, M. 1995.** Bioassay and preliminary characterization of ovigerous-hair stripping substance (OHSS) in hatch water of crab larvae. *Biol. Bull.* **189**: 175–184.
- Saigusa, M. 1996.** Two kinds of active factor in crab hatch water: ovigerous-hair stripping substance (OHSS) and protease. *Biol. Bull.* **191**: 234–240.

- Saigusa, M. 1997.** Hatching and its control by the circatidal clock in marine crustaceans. *Proc. Arthropod. Embryol. Soc. Jpn.* **32**: 1–23.
- Yamagami, K. 1988.** Mechanisms of hatching in fish. Pp. 447–499 in *Fish Physiology*, Vol. 11A, W. S. Hoar and D. J. Randall, eds. Academic Press, San Diego, California.
- Yasumasu, S., I. Iuchi, and K. Yamagami. 1989a.** Purification and partial characterization of high choriolytic enzyme (HCE), a component of the hatching enzyme of the teleost, *Oryzias latipes*. *J. Biochem.* **105**: 204–211.
- Yasumasu, S., I. Iuchi, and K. Yamagami. 1989b.** Isolation and some properties of low choriolytic enzyme (LCE), a component of the hatching enzyme of the teleost, *Oryzias latipes*. *J. Biochem.* **105**: 212–218.
- Yasumasu, S., S. Katow, T. S. Hamazaki, I. Iuchi, and K. Yamagami. 1992.** Two constituent proteases of a teleostean hatching enzyme: concurrent syntheses and packaging in the same secretory granules in discrete arrangement. *Dev. Biol.* **149**: 349–356.
- Yonge, C. M. 1937.** The nature and significance of the membranes surrounding the developing eggs of *Homarus vulgaris* and other Decapoda. *Proc. Zool. Soc. Lond. (Ser. A)*. **107**: 499–517.
- Yonge, C. M. 1946.** Permeability and properties of the membranes surrounding the developing egg of *Homarus vulgaris*. *J. Mar. Biol. Assoc. UK* **26**: 432–438.